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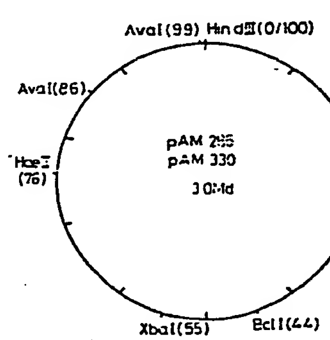
(54) Plasmid.

(57) An essentially pure plasmid is provided, which is characterized by a molecular weight of 3.0 ± 0.1 megadalton and a restriction endonuclease cleavage chart according to Figure 1.

This plasmid may be inserted with a gene which is responsible for the genetic information for the production of a product and the hybrid plasmid may be incorporated into Coryneform glutamic acid producing bacteria as recipient organisms in order to improve their characteristics.

Figure 1



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PLASMID

The present invention relates to a plasmid, particularly to a plasmid capable of propagating in Coryneform glutamic acid-producing bacteria.

Coryneform glutamic acid producing bacteria belong to so called "Coryneform" bacteria, and are known to produce high amounts of L-glutamic acid, and mutants of the Coryneform glutamic acid-producing bacteria produce amino acid such as lysine and purine nucleotide such as inosinic acid. Therefore, they are of great importance for the fermentation industry.

The recently developed gene splicing techniques can successfully be applied for producing or improving industrial microorganisms, especially in case of Escherichia coli. It has been difficult, however, to apply the gene splicing techniques for producing or improving industrial microorganisms of such Coryneform glutamic acid-producing bacteria, since suitable plasmids useful for the construction of such industrial microorganisms of Coryneform glutamic acid-producing bacteria have not yet been found.

A plasmid capable of propagating in Coryneform glutamic acid-producing bacteria was reported in Agric. Biol. Chem., 43, 867, (1979), however, the

molecular weight of the known plasmid is 37 megadalton and is too large and inconvenient to use for the construction of industrial microorganisms of Coryneform bacteria by gene splicing technique. Therefore, a continuous need exists for the development of a novel plasmid useful for constructing or improving industrial microorganisms from Coryneform glutamic acid-producing bacteria.

It is therefore an object of the present invention to provide a novel plasmid useful for producing or improving industrial microorganisms from Coryneform glutamic acid-producing bacteria.

This and other objects of the present invention have been attained by providing an

essentially pure plasmid which is characterized by a molecular weight of 3.0 ± 0.1 megadalton and a restriction endonuclease cleavage chart shown in Figure 1.

Specimens of the plasmid of the present invention are pAM 330 separated from Brevibacterium lactofermentum ATCC 13869, and pAM 286 separated from Corynebacterium glutamicum AJ 11560 (FERM-P 5485). These plasmids have the following common characteristics.

Molecular weights calculated by the migration distance in agarose gel electrophoresis and by the length of the DNA-chain under an electron microscope are both 3.0 megadalton. The sensitivity to restriction enzymes and restriction charts of the two plasmids are the same and shown in Table 1 and Figure 1, respectively.

The plasmids pAM 330 and pAM 286 can be obtained from the cells of the deposited microorganisms Brevibacterium lactofermentum ATCC 13869 and Corynebacterium glutamicum FERM-P 5485 (NRRLB-12415), respectively, by lysing the cells previously harvested at a late exponential growth phase by lysozyme and SDS, adding polyethylene glycol to the supernatant obtained from the lysate by centrifugation at 30,000 xg, and purifying the precipitated DNA by fractionation by means of cesium chloride-ethidium bromide equilibrium density gradient centrifugation.

TABLE 1

Restriction Enzyme		Number of Restriction Site
Alu I	Arthrobacter luteus	≥ 4
Ava I	Anabena variabilis	≥ 2
Bcl I	Bacillus caldolyticus	1
BamH I	Bacillus amyloliquefaciens H	0
Bgl II	Bacillus globigii	0
BstE II	Bacillus stearothermophilus ET	≥ 4
EcoR I	Escherichia coli RI+	0
Hae II	Haemophilus aegyptius	1
HgiA I	Herpetosiphon giganteus	≥ 4

	Restriction Enzyme	Number of Restriction Site
Hind II	Haemophilus influenzae	≥ 4
Hind III	Haemophilus influenzae	1
Hpa II	Haemophilus parainfluenzae	≥ 4
Kpn I	Klebsiella pneumoniae	0
Pvu II	Proteus vulgaris	0
Sac I	Streptomyces achromogenes	0
Sal I	Streptomyces albus	0
Sau 3A	Staphylococcus aureus	≥ 4
Sma I	Serratia marcescens	1
Sst I	Streptomyces stanford	0
Xba I	Xanthomonas badrii	1
Xho I	Xanthomonas holicola	1
Xma I	Xanthomonas malvacearum	1
Xor II	Xanthomonas oryzae	0

Coryneform bacteria are aerobic, gram-positive rods, non-sporulating, and non-acidfast, and are described in Bergey's Manual of Determinative Bacteriology, 8th ed., 599, (1974). Examples of wild strains of Coryneform glutamic acid-producing bacteria useful as hosts in this invention are as follows:

Brevibacterium divaricatum ATCC 14020,

Brevibacterium saccharoliticum ATCC 14066,

Brevibacterium immariophilum ATCC 14068,

Brevibacterium lactofermentum ATCC 13869,
Brevibacterium roseum ATCC 13825,
Brevibacterium flavum ATCC 13826,
Brevibacterium thiogenitalis ATCC 19240,
Corynebacterium acetoacidophilum ATCC 13870,
Corynebacterium acetoglutamicum ATCC 15806,
Corynebacterium callunae ATCC 15991,
Corynebacterium glutamicum ATCC 13032, 13060
Corynebacterium lilium ATCC 15990,
Corynebacterium melassecola ATCC 17965,
Microbacterium ammoniaphilum ATCC 15354.

Coryneform glutamic acid-producing bacteria also include mutants which have lost the productivity for glutamic acid or have productivity of other amino acids such as lysine and arginine; purine nucleosides such as inosine; purine nucleotides such as inosine-5'-monophosphate; and other fermentation products.

In cells of those Coryneform glutamic acid-producing bacteria, the plasmids of the present invention are stably maintained. Since the plasmid of the present invention can propagate in cells of Coryneform glutamic acid-producing bacteria, the information of a foreign gene inserted in the plasmid can be amplified in the hosts.

The incorporation of plasmid DNA into Coryneform glutamic acid producing bacteria as hosts can be done by treating the cells of the DNA-recipient with calcium chloride

to increase the permeability for DNA, as is reported regarding E. coli K-12 (Mandel, M. and Higa, A., J. Mol. Biol., 53, 159 (1970)), or by incorporating at a specific a stage of growth when cells become capable of incorporating DNA (competent cells) as is reported in Bacillus subtilis (Duncan, C. H., Wilson, G. A. and Young, F. E., Gene 1, 153 (1977)).

The plasmid can also be incorporated into the DNA-recipient by forming protoplasts or spheroplasts of the DNA-recipient, which easily incorporate plasmid DNA, as is known in Bacillus subtilis, actinomycetes and yeast (Chang, S. and Choen, S. N., Molec. Gen. Genet., 168, 111 (1979)); Bibb, M. J., Ward, J. M. and Hopwood, O. A., Nature, 274, 398 (1978); Hinnen, A., Hicks, J. B. and Fink, G. R., Proc. Nat 1. Acad. Sci., USA. 75, 1929 (1978)).

A foreign gene can be inserted into the plasmid of the present invention at the position cleaved by restriction enzymes shown in Table 1 and more preferably at the cleavage position whose number is one. As the foreign gene, genom DNA of Coryneform glutamic acid-producing bacteria is the most preferred. A preferred vector can be obtained from the present plasmid by removing all or a part of DNA regions except the drive-ur (vehicle unit), since the molecular weight becomes small. The replication number of the present plasmid is sufficiently large and therefore is suitable for amplifying foreign gene.

Example 1

Preparation of pAM 330 DNA

Brevibacterium lactofermentum ATCC 13869 which possessed pAM 330 was cultured at 30°C in CMG medium of pH 7.2 containing 1 g/dℓ peptone, 1 g/dℓ yeast extract, 0.5 g/dℓ glucose and 0.5 g/dℓ glucose and 0.5 g/dℓ NaCℓ until the late exponential growth phase. Cells were harvested and lysed by treatment with lysozyme and SDS. The lysate was centrifuged to obtaina supernatant, which was added with polyethylene glycole and evaporated to precipitate the DNA. The precipitate was dissolved in tris-EDTA-NaCℓ buffer of pH 8.0, and the solution was subjected to agarose-gel electrophoresis (5 v/cm, 15 hours). Thus, 74 μg of pAM 330 plasmid DNA was obtained.

Molecular weight of pAM 330

The molecular weight of pAM 330 was calculated from the migration distance on agarose gel electrophoresis and the length of the DNA-chain under an electron microscope. The agarose gel electrophoresis was carried out according to P.A. Sharp's method(Biochemistry 12, 3055 (1973)),(0.7 - 0.8% gel, 5 v/cm, and 15 hours). The molecular weight was calculated based on the mobility differences of pBR 322 (Boliver F. et al; Gene, 2, 95, (1977)), pUB 110 (Gryczan T. J. et al; J. Bacteriol, 134, 318, (1978)) and Col E1 (Bazaral M. et al; J. Mol. Biol. 36, 185, (1968)).

The Cytochrome C monolayer method (Kleinschmidt A, Zahn R. K.; Z.Naturforsh, 14b, 770 (1959)) was applied for the electron microscope observation.

Agarose-gel electrophoresis of the digested plasmid

Commercially available restriction enzymes sold by Bethesd Research Laboratory Co., Boehringer Mannheim Co., New England Biolab Co., Worthington Biochemical Co. were used. The digestion by restriction enzyme was carried out using an excessive amount of restriction enzyme, e.g. more than 3 times of the amount required. Digestion reaction was carried out according to the well-known directions by the distributors. When the digestion was done by using more than two restriction engymes, the DNA fragments formed by the last digest reaction were separated by the method described in T. Tanaka, B. Weisblum; J. Bacteriol., 121, 354 (1975) and precipitated by ethanol. Thereafter the separated DNA fractions were subjected to the next digestion reaction.

The DNA fragments were thereafter subjected to agarose-gel electrophoresis. The Agarose concentration was 0.7 to 1%, the voltage was 5 to 20 v and the electrophoresis was continued for 1 to 3 hours. The molecular weight was calculated based on the mobility difference between the DNA fragments and authentic samples (ø X 174 RF-Hae III fragment and λ DNA-Hind III fragment both produced and sold by Bethesda Research Laboratory).

Determination of the replication number of pAM 330

Brevibacterium lactofermentum ATCC 13869 containing pAM 330 was cultured at 30°C overnight in 5 mℓ of a minimal medium of pH 7.0 containing per liter, 20 g glucose, 10 g $(NH_4)_2SO_4$, 2.5 g urea, 1 g KH_2PO_4, 0.4 g $MgSO_4 \cdot 7H_2O$, 50 μg biotin, 200 μg thiamine·HCℓ, 0.01 g $FeSO_4 \cdot 7H_2O$ and 0.01 g $MnSO_4 \cdot 4H_2O$, and

further added with 100 μCi of ^{3}H-thymidine. The cells obtained were lysed by lysozyme and SDS, and circular plasmid was separated by cesium chloride-ethidium bromide equilibrium density gradient centrifugation.

The radioactivity recovery in the circular plasmid was 0.8 to 1.0 %. Since the molecular weight of pAM 330 was $3X10^6$ and that of chromosomal DNA of ATCC 13869 was $3X10^9$, the copy number of the circular plasmid was calculated as 8 to 10. (Only circular plasmid can be determined by the method above and plasmid in cell and plasmid which became linear are not included, and therefore actual copy number may be larger than that determined (Kenji Nagahari; BUNSHI IKUSHU TO OHYOBISEIBUTSU, Kodansha,p172, (1979)).

Preparation of recombinant DNA from pAM 330

Brevibacterium lactofermentum No. 5116 (NRRL B-12405), a mutant sensitive to a high temperature and induced from strain No. 2256 (ATCC 13869), was cultured at 30°C for 3 hours with shaking in 1ℓ of CMG-medium containing 1g/dℓ peptone, 1g/dℓ yeast extract, 0.5g/dℓ glucose and 0.5g/dℓ NaCℓ (pH was adjusted to 7.2), and bacterial cells in the exponential growth phase were harvested. Chromosomal DNA was extracted by a conventional phenol-method, and 3.5mg of purified DNA was obtained.

Ten μg of the chromosomal DNA was treated with each of the restriction endonucleases Hind III at 37°C for 10, 30 and 60 minutes respectively, to cleave DNA chains, and then was heated at 65°C for 5 minutes. Five μg of pAM 330 DNA was also treated

with Hind III at 37°C for 1 hour to cleave the DNA completely, and then was heated at 65°C for 5 minutes.

The digested chromosomal DNA solution and the cleaved vector DNA solution were mixed and subjected to the ligation reaction of DNA fragments by a T_4 phage DNA-ligase in the presence of ATP and dithiothreitol at 10°C for 24 hours. The reaction mixture was then heated at 65°C for 5 minutes, and the two fold volume of ethanol was added to it. The recombinant DNA which precipitated was recovered.

A glutamic acid requiring strain of Brevibacterium lactofermentum No. 3 (NRRL B-12406) which was derived from Brevibacterium lactofermentum No. 5116 by N-methyl-N'-nitro-N-nitrosoguanidine mutagenesis, was cultured in 20 mℓ of CMG at 30°C with shaking. Cells in the exponential growth phase were harvested, and suspended in a 0.1M $MgCl_2$ solution and then in a 0.1M $CaCl_2$ solution in an ice-bath, whereby, "competent" cells having the ability of DNA uptake were prepared.

Into the competent cell suspension, the recombinant DNA was added. The suspension was kept in an ice-bath for 30 minutes, then heated at 42°C for 2 minutes, and again allowed to stand in an ice-bath for 30 minutes. The cells, thus containing the hybrid plasmid DNA, were inoculated into an L-medium and the medium was shaken at 37°C for 3 hours, whereby the transformation reaction was completed. The cells were harvested, washed, and resuspended. A small portion of the cell suspension was spread on an agar plate containing, 20g

glucose, 10g $(NH_4)_2SO_4$, 2.5g urea, 1g KH_2PO_4, 0.4g $MgSO_4 \cdot 7H_2O$, 50 μg biotin, 200 μg thiamine hydrochloride, 0.01g $FeSO_4 \cdot 7H_2O$, 0.01g $MnSO_4 \cdot 4H_2O$ and 20 agar, per liter, (pH was adjusted to 7.2). The plate was incubated at 37°C. After 4 days incubation, all of the colonies which appeared were picked up, purified and isolated.

Strains which became capable of producing L-glutamic acid by the transformation were picked up as the transformants. Among the transformants, most high L-glutamic acid producer AJ 11561 (FERM-P 5469) (NRRL B-12408) was selected.

The L-glutamic acid productivity of AJ 11561 was tested comparing with the DNA-donor and the recipients, as follows:

The fermentation medium contained 3.6g/dℓ glucose, 0.5g/dℓ urea, 0.1g KH_2PO_4, 0.1g/dℓ $MgSO_4 \cdot 7H_2O$, 3 mℓ/dℓ soybean hydrolysate ("Mieki"), 100 μg/ℓ thiamine·HCℓ 3 μg/ℓ biotin, 1 mg/dℓ $FeSO_4 \cdot 7H_2O$, 1 mg/dℓ $MnSO_4 \cdot 4H_2O$ and 2.5g/dℓ $CaCO_3$ (separately sterilized) and the pH was adjusted to 7.0.

Twenty mℓ of the fermentation medium was placed in 500 mℓ flasks, inoculated with one loopful inoculum of the test microorganisms, and the cultivation was performed at 31°C for 48 hours.

The amounts of L-glutamic acid in the supernatant of the fermentation broth were determined by enzymatic assay.

TABLE 1

Microorganisms tested	Amounts of L-glutamic acid accumulated (mg/dℓ)
Brevibacterium lactofermentum No. 5116	550
Brevibacterium lactofermentum No. 3	0
Brevibacterium lactofermentum AJ11561	980

Example 2

(1) Preparation of chromosomal DNA possessing genetic information related to L-glutamic acid production

Corynebacterium glutamicum No. 5707 (NRRLB- 12410), a mutant resistant to ketomalonic acid and induced from Corynebacterium glutamicum AJ 11560 (FERM-P 5485) (NRRL B-12415), was cultured at 30°C for 3 hours with shaking in 1ℓ of CMG-medium containing 1g/dl peptone, 1g/dl yeast extract, 0.5g/dl glucose and 0.5g/dl NaCl (pH was adjusted to 7.2), and bacteria cells in the exponential growth phase were harvested. Chromosomal DNA was extracted by a conventional phenol-method, and 4.0 mg of purified DNA was obtained.

Corynebacterium glutamicum AJ 11560 was newly isolated as a suitable strain for the purpose of this invention.

This strain, AJ 11560, was classified into the section III of genus Corynebacterium described in Bergey's Manual of Determinative Bacteriology (8th edition, 1974). However, taxonomic characteristics of the species belonging to section III are not disclosed in the Manual, disclosed are only the names of species of section III. Therefore, all original reports disclosed in the Manual as to section III are referred to. AJ 11560 was identified as Corynebacterium glutamicum described in "Bull. Agr. Chem. Soc. Japan, 22, 176-185 (1958)" and "J. Gen. Appl. Microbiol., 13, 279-301 (1967)".

(2) Preparation of vector DNA

As the vector, the DNA of plasmid PAM286 (M.W. $3x10^6$ dalton) was prepared as follows:

A strain of Corynebacterium glutamicum AJ 11560 harboring the plasmid pAM286 was incubated at 30°C in 1ℓ of CMG-medium. After the strain was incubated until the late log phase, the cells were harvested and then lysed by treatment with lysozyme and SDS. The lysate was centrifuged at 30,000 Xg for 30 minutes to obtain a supernatant. After concentrating the supernatant, 60 μg of the plasmid DNA was obtained by fractionation using agarose gel electrophoresis.

(3) <u>Insertion of chromosomal DNA fragment into the vector</u>

Ten μg of the chromosomal DNA was treated with each of the restriction endonucleases Hind III or Xma I at 37°C for 10, 30 and 60 minutes respectively, to cleave DNA chains, and then was heated at 65°C for 5 minutes, respectively. Ten μg of the vector DNA was also treated with each of the restriction endonucleases, Hind III or Xma I at 37°C for 1 hour to cleave the DNA completely, and then was heated at 65°C for 5 minutes, respectively.

The digested chromosomal DNA solution and the cleaved vector DNA solution were mixed and subjected to the ligation reaction of DNA fragments by a T_4 phage DNA-ligase in the presence of ATP and dithiothreitol at 10°C for 24 hours. The reaction mixture was then heated at 65°C for 5 minutes, and the two fold volume of ethanol was added to it. The recombinant DNA which precipitated was recovered.

(4) Genetic transformation with the hybrid plasmid harboring the genetic information related to glutamic acid production

L-Glutamic acid requiring strains of Corynebacterium glutamicum No. 12 (NRRL B-12411) and No. 26 (NRRL B-12412), which were derived from Corynebacterium glutamicum No. 5707 by N-methyl-N'-nitro-N-nitrosoguanidine mutagenesis, were cultured in 20 ml of CMG - medium at 30°C with shaking. Cells in the exponential growth phase were harvested, and suspended in a 0.1M $MgCl_2$ solution and then in a 0.1M $CaCl_2$ solution in an ice-bath, whereby "competent" cells having the ability of DNA uptake were prepared.

Into the competent cell suspension, the DNA obtained in step (3), which contains the hybrid plasmid DNA, was added. The suspension was kept in an ice-bath for 30 minutes, then heated at 42°C for 2 minutes, and again allowed to stand in an ice-bath for 30 minutes. The cells, thus containing the hybrid plasmid DNA, were inoculated into an L-medium and the medium was shaken at 37°C for 3 hours, whereby the transformation reaction was completed. The cells were harvested, washed, and resuspended. The reaction mixture, after having been diluted, of the cell suspension was spread on an agar plate containing, 20g glucose, 10g $(NH_4)_2SO_4$, 2.5g urea, 1g KH_2PO_4, 0.4g $MgSO_4 \cdot 7H_2O$, 50 μg biotin, 20 μg thiamine hydrochloride, 0.01g $FeSO_4 \cdot 7H_2O$, 0.01g $MnSO_4 \cdot 4H_2O$ and 20g agar, per liter, (pH was adjusted to 7.0). The plate was incubated at 37°C. After 4 days incubation, all of the colonies which appeared were picked up, purified and isolated.

AJ 11566 (FERM-P 5486) (NRRL B-12413) from the recipient strain No. 12 using Hind III, and AJ 11567

(FERM-P 5487) (NRRL B-12414) was obtained from the recipient strain No. 26 using Xma I.

(5) Production of L-glutamic acid by the prepared glutamic acid producing strain

The transformants obtained in step (4) were cultured to test their L-glutamic acid productivity. The DNA-donor strain No. 5707 and the recipients strains were cultured in the same manner for comparison.

The culture medium contained 3.6g/dl glucose, 0.5g/dl urea, 0.1g KH_2PO_4, 0.1g/dl $MgSO_4 \cdot 7H_2O$, 3 ml/dl soybean hydrolysate ("Mieki"), 100 µg/l thiamine·HCl 3 µg/l biotin, 1 mg/dl $FeSO_4 \cdot 7H_2O$, 1 mg/dl $MnSO_4 \cdot 4H_2O$ and 2.5g/dl $CaCO_3$ (separately sterilized) and the pH was adjusted to 7.0.

Twenty ml of the fermentation medium was placed in a 500 ml flasks, inoculated with one loopful inoculum of the test microorganisms, and the cultivation was performed at 31°C for 48 hours.

The amounts of L-glutamic acid in the supernatant of the fermentation broth were determined by enzymatic assay.

TABLE 1

Microorganisms tested	Amounts of L-glutamic acid accumulated (mg/dl)
Corynebacterium glutamicum No. 5707	600
Corynebacterium glutamicum No. 12	0
Corynebacterium glutamicum No. 26	0
Corynebacterium glutamicum AJ 11566	1010
Corynebacterium glutamicum AJ 11567	1000

Example 2

Preparation of pAM 286 DNA

Corynebacterium glutamicum AJ 11560 (FERM-P 5485) which contains pAM 286 DNA was cultured in CMG medium at 30°C until the late exponential growth phase. Cells harvested were lysed by lysozyme and SDS treatment. The supernatant of the lysate was separated by centrifugation at 30,000 xg for 30 minutes, and added with polyethyleneglycol to precipitate DNA. The precipitate was dissolved in 1/10 volume of TEN buffer of pH 8.0 containing 20 mM Tris, 20 mM NaCl and 1 mM of EDTA, and thereafter circular plasmid was separated by cesium chloride-ethidium bromide equilibrium density gradient centrifugation. Ethidium bromide was removed from the circular plasmid fraction which was thereafter subjected to dialysis purification, whereby 82 μg of pAM 286 DNA was obtained. The above plasmid DNA isolation procedure was in conformity with the disclosure of Tamio Yamakawa (ed.); SEIKAGAKU JIKKEN KOZA Vol 1, (1) 73, Tokyo Kagaku Donin (1975).

Digestion with restriction enzyme and agarose-gel electro phoresis were performed following the procedure shown in Example 1.

The determination of copy number of pAM 286 was carried out in the manner shown in Example 1 using Corynebacterium glutamicum AJ 11560 as the host, and the copy number was 8 to 10.

Determination of molecular weight of pAM 286

The molecular weight of pAM 286 was determined by the agarose-gel electrophoresis and electron microscope observation

shown in Example 1.

Preparation of recombinant DNA from pAM 286

Corynebacterium glutamicum No. 22 (NRRL B-12416), a mutant resistant to S-(2-aminoethyl)-cystein (AEC) and induced from Corynebacterium glutamicum AJ 11560 (FERM-P 5485) (NRRL B-12415), was cultured at 30°C for 3 hours with shaking in 1ℓ of CMG-medium containing 1g/dℓ peptone, 1g/dℓ yeast extract, 0.5g/dℓ glucose and 0.5g/dℓ NaCℓ (pH was adjusted to 7.2), and bacterial cells in the exponential growth phase were harvested. Chromosomal DNA was extracted by a conventional phenol-method, and 4.0mg of purified DNA was obtained.

Ten μg of the chromosomal DNA was treated with the restriction endonuclease XbaI at 37°C for 10, 30 or 60 minutes, to cleave DNA chains, and then was heated at 65°C for 5 minutes, respectively. Five μg of the vector DNA was also treated with the restriction endonuclease, XbaI at 37°C for 1 hour to cleave the DNA completely, and then was heated at 65°C for 5 minutes.

The digested chromosomal and vector DNAs were mixed and subjected to the ligation reaction by T_4 DNA-ligase in the presence of ATP and dithiothreitol at 10°C for 24 hours. The reaction mixture was then heated at 65°C for 5 minutes, and two fold volumes of ethanol were added to it. The recombinant DNA which precipitated was recovered.

A L-lysine requiring strain, Corynebacterium glutamicum No. 97 (NRRL B-12417), which was derived from Corynebacterium glutamicum No. 22 by N-methyl-N'-nitro-N-nitrosoguanidine

mutagenesis, was cultured in 20mℓ of CMG - medium at 30°C with shaking. Cells in the exponential growth phase were harvested, and "competent" cells having the ability of DNA uptake were prepared by the $CaC\ell_2$-treatment.

Into the competent cell suspension, the DNA obtained was added, and the DNA was incorporated into the cells. After the transformation reaction, the cell suspension was spread on an agar plate containing, 20g glucose, 10g $(NH_4)_2SO_4$, 2.5g urea, 1g KH_2PO_4, 0.4g $MgSO_4 \cdot 7H_2O$, 50 μg biotin, 200 μg thiamine hydrochloride, 0.01g $FeSO_4 \cdot 7H_2O$, 0.01g $MnSO_4 \cdot 4H_2O$, 3.0g AEC·HCℓ and 20g agar, per liter, (pH was adjusted to 7.0). The plate was incubated at 30°C. After 4 days incubation, all of the colonies, which appeared and got the productivity of L-lysine and resistance to AEC, were picked up, purified and isolated. Thus, AJ 11575 (FERM-P 5501) (NRRL B-12418) was obtained.

The transformants obtained were cultured to test their L-lysine productivity. The DNA-donor strain No. 22 and the recipients strain No. 97 were cultured in the same manner for comparison.

The culture medium contained 10g/dℓ glucose, 0.5g/dℓ urea, 4.5g/dℓ $(NH_4)_2SO_4$, 0.1g/dℓ KH_2PO_4, 0.04g/dℓ $MgSO_4 \cdot 7H_2O$, 10mg/dℓ adenine, 10mg/dℓ sodium glutamate, 0.1mg/ℓ thiamine·HCℓ 0.5mg/ℓ biotin, 1mg/dℓ $FeSO_4 \cdot 7H_2O$, 10mg/dℓ $MnSO_4 \cdot 4H_2O$ and 5g/dℓ $CaCO_3$ (separately sterilized) and the pH was adjusted to 8.0.

Twenty mℓ batches of the fermentation medium were placed in 500mℓ flasks, inoculated with one loopful inoculum of the test microorganisms, and the cultivation was performed at 31°C for 70 hours.

The amounts of L-lysine in the supernatant of the fermentation broth were determined by micro-biological assay.

TABLE 1

Microorganisms tested	Amount of L-lysine accumulated (mg/dℓ)
Corynebacterium glutamicum No. 22	120
Corynebacterium glutamicum No. 97	12
Corynebacterium glutamicum AJ 11575	235

In this specification the term ATCC is used for the deposition at American Type Culture Collection, U.S.A., NRRL for the deposition at Agricultural Research Culture Collection, U.S.A. and FERM for the deposition at Fermentation Research Institute, Agency of Industrial Science and Technology, Ministry of International Trade and Industry, Japan.

CLAIMS

1. A substantially pure plasmid which is characterized by a molecular weight of 3.0 $\pm$ 0.1 megadalton and a restriction endonuclease-cleavage chart shown in Figure 1.

2. A substantially pure plasmid of Claim 1, which is pAM 330.

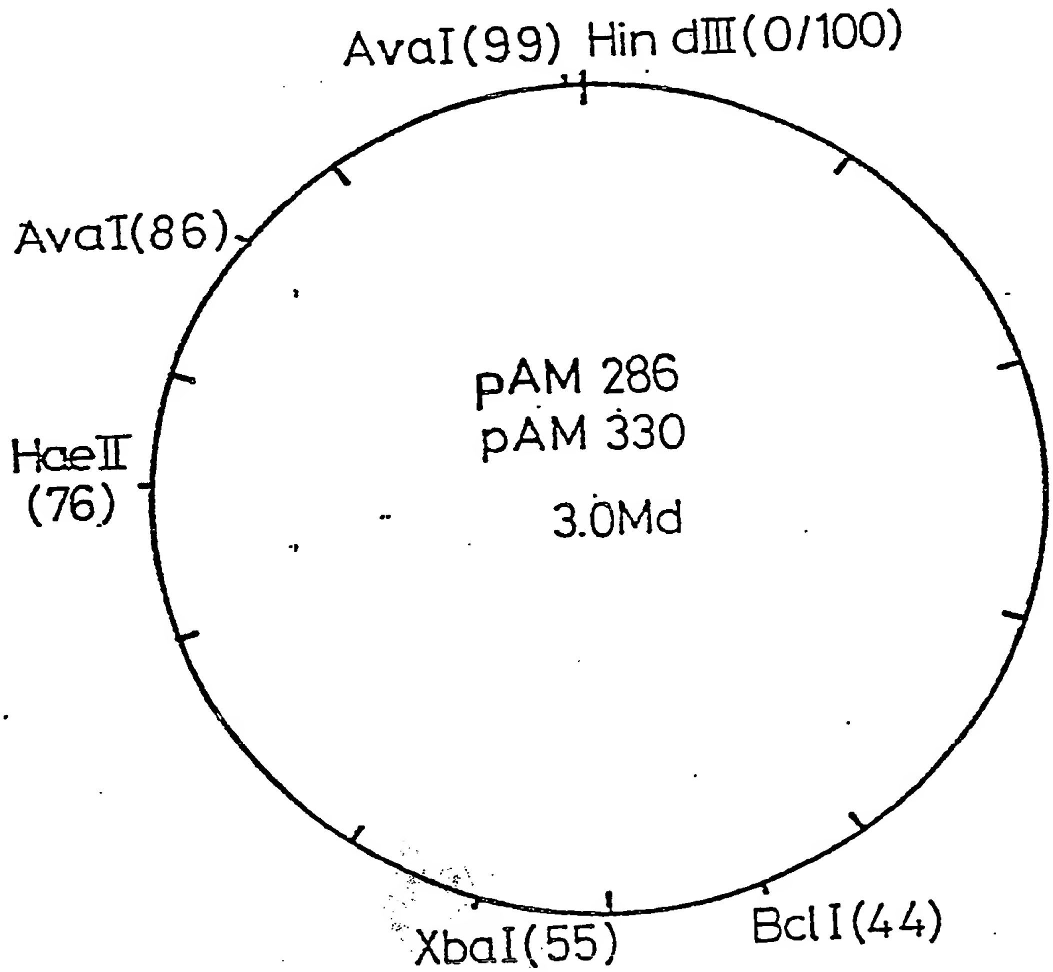
3. A substantially pure plasmid of Claim 1, which is pAM 286.

4. A substantially pure plasmid obtained by insertion of a foreign gene into a plasmid, which is characterized by a molecular weight of 3.0 $\pm$ 0.1 megadalton and a restriction endonuclease-cleavage chart shown in Figure 1.

5. A process for producing a microorganism from Coryneform glutamic acid producing bacteria , which comprises incorporating into a Coryneform glutamic acid producing bacterium as a recipient a plasmid into which a gene is inserted, which is responsible for the genetic information for the production of a product, characterized in that the plasmid according to claim 4 is incorporated into the recipient.

1/1

Figure 1


AvaI(99)
Hin dIII(0/100)
AvaI(86)
HaeII
(76)
pAM 286
pAM 330
3.0Md
XbaI(55)
BclI(44)